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The role of CELF proteins in neurological disorders

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Abbreviations: ALS, amyotrophic lateral sclerosis; CELF, CUG-BP and ETR-3-like factors; DM1, myotonic dystrophy, type 1; FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17; FXTAS, fragile-X-associated tremor/ataxia syndrome; GAP, GTPase activating protein; MBNL1, muscleblind-like 1; NF1, neurofibromatosis type 1; RRM, RNA recognition motif; SCA, spinocerebellar ataxia; SMA, spinal muscular atrophy; SMN, survival motor neuron; UTR, untranslated region

CELF (CUG-BP and ETR-3-like factors) proteins are structurally related RNA-binding proteins involved in various aspects of RNA processing including splicing and mRNA stability. The first member of the family, CELF1/CUG-BP1, was identified through its role in myotonic dystrophy, type 1. Several recent studies have uncovered the recurrent implication, to various extents, of CELF proteins or of the functionally related muscleblind-like 1 protein in a number of neurological conditions. This is particularly clear for inherited neurodegenerative disorders caused by expansions of translated or untranslated triplet repeats in the causative gene. Here we review the role played by CELF proteins, at least as modifiers of the pathological phenotype, in a number of neurological diseases. The involvement of CELF proteins suggest that individual pathogenic pathways in a number of neurological conditions overlap at the level of RNA processing.

Introduction

An increasing number of neurological disorders are being linked to impairment of RNA processing.[1-5] Impaired RNA processing can be caused by mutations in cis-acting elements or can result from abnormal activity of RNA-binding proteins. A prominent example of the former is provided by dominant mutations in the *MAPT* gene, encoding the microtubule-associated protein, tau, in frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The RNA- and DNA-binding protein, TDP-43, accumulates in characteristic cytoplasmic inclusions in amyotrophic lateral sclerosis (ALS), and mutations in the *TARDBP* gene, encoding TDP-43, as well as in the *FUS* gene, encoding another RNA-binding protein, FUS, have been linked to familial forms of ALS.[6] While not a primary cause, aberrant RNA processing has been shown to be part of altered molecular pathways involved in many neurological disorders. Here we review the role played by a group of RNA-binding proteins, the CELF proteins, at least as modifiers of the pathological phenotype, in a number of neurological conditions, particularly in neurodegenerative disorders caused by triplet repeat expansions.

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The CELF Family of RNA-Binding Proteins

CELF proteins (CUG-BP and ETR-3-like factors) proteins are a family of structurally related RNA-binding proteins involved in various aspects of RNA processing. The first member of the CELF family, CUG-BP1, was identified in investigating the molecular mechanisms of myotonic dystrophy. Myotonic dystrophy, type 1 (DM1) is an inherited progressive muscle wasting disease caused by a CTG repeat expansion from 50 to over 1,000 in the 3'-untranslated region (UTR) of the DM protein kinase (*DMPK*) gene. The molecular mechanisms of DM1 have been reviewed extensively elsewhere.[7-9] A search for proteins binding to repeated CUG sequences identified a ~50 kDa protein, CUG-BP1, binding to single-stranded CUG repeated sequence.[10,11] A comprehensive search of human expressed sequence tag databases for proteins structurally related to CUG-BP1 identified a family of six proteins.[12,13] One such protein is ETR-3 (ELAV-type RNA-binding protein 3), related to Drosophila embryonic lethal abnormal vision protein (ELAV). Members of this family are now referred to as CELF (CUG-BP and ETR-3-like factors) proteins. Independently, a similar search for mammalian homologues of the *Xenopus laevis* BrunoL-1 protein identified the same family of RNA-binding proteins, thus also referred to as Bruno-like (BRUNOL) family.[14] In Drosophila, the Bruno protein represses the translation of the *oskar* mRNA, required in early embryonic development. The structure of the six known members of the CELF family is compared in **Figure 1**. CELF proteins have been referred to by their CELF or BRUNOL nomenclature, or by specific names (**Table 1**). The CELF nomenclature will be used throughout this review.

CELF proteins have three conserved RNA recognition motifs (RRM), RRM1 and RRM2 in the N-terminus of the protein and RRM3 in the C-terminus. RRM3 is separated from RRM1 and RRM2 by a non-conserved domain, or divergent domain, specific for each member of the family.[12] Each

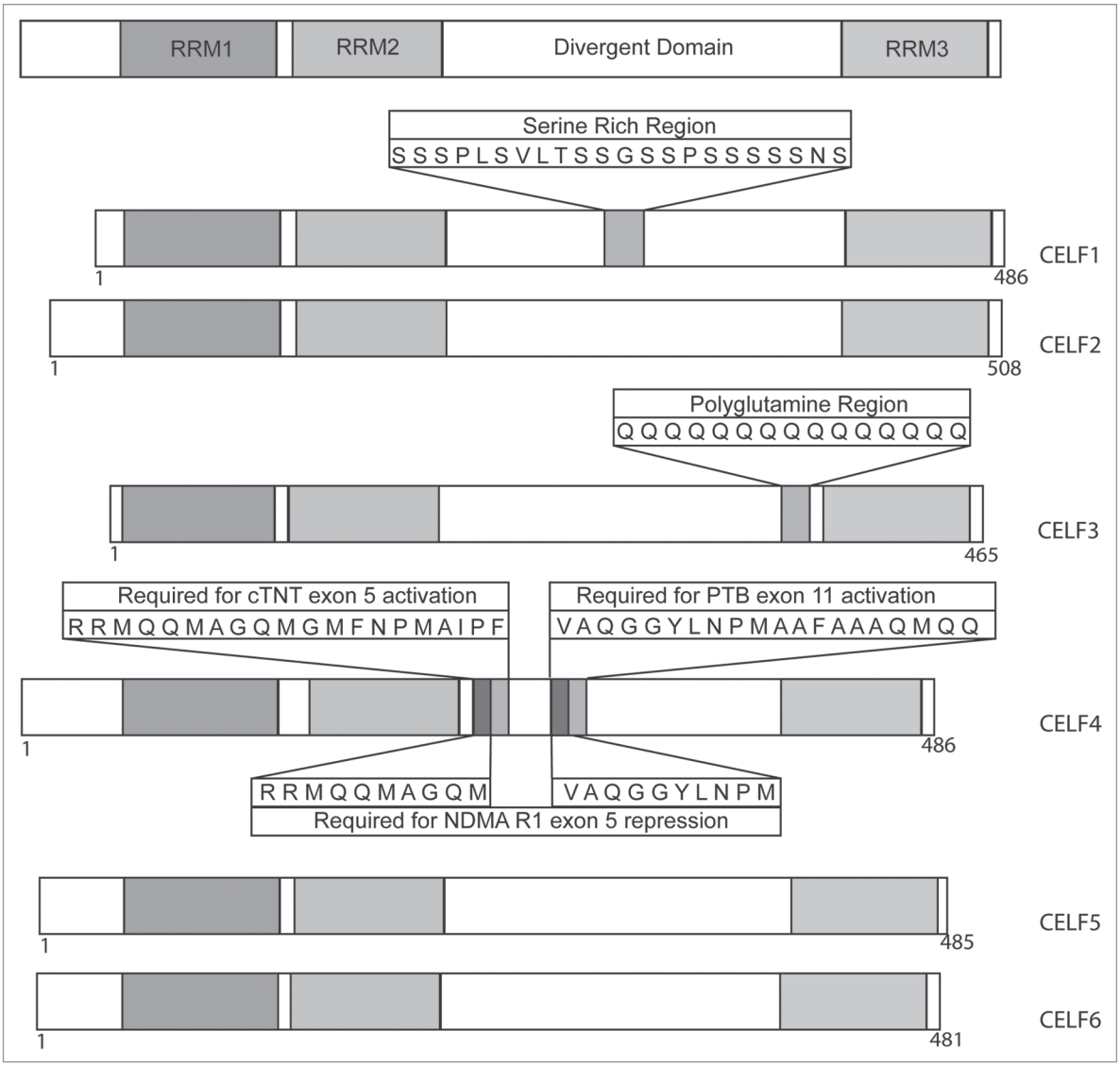

Figure 1. Comparison of the structure of the six members of the human CELF protein family.[12-14] CELF proteins have a similar structure comprising three conserved RRMs, two in the N-terminus, RRM1 and RRM2 (left as shown on top bar), and a third, RRM3 (right as shown on top bar) in the C-terminus. A non conserved domain, the divergent domain, separates RRM3 from RRM2. Notable differences between the divergent domains of the CELF proteins are a serine-rich region in CELF1 (identified by primary sequence comparison), a polyglutamine stretch in CELF3,[12,26] and specific regions in the divergent domain of CELF4 which modulate its splicing activity on different pre-mRNA targets.[71]

RRM comprises two ribonucleoprotein motifs, (RNP1 and RNP2) responsible for RNA binding; RNP2 is highly conserved among CELF proteins.[12,14] Unique among other CELF proteins is CELF3, that contains a 15–18-repeat polyglutamine (polyQ) sequence in its divergent domain. CELF proteins usually bind to single stranded CUG/GUG clusters in introns or in 3'-UTRs of mRNAs.[15,16] CELF2 has been implicated in cytoplasmic functions, such as translational control and mRNA stability[17] as well as RNA editing.[18] CELF1 regulates alternative splicing of several transcripts known to be misregulated in DM1 including cardiac troponin T, the insulin receptor, and the muscle-specific chloride channel.[7]

In DM1, the CUG expansion in the 3'-UTR of the *DMPK* mRNA activates protein kinase C (PKC), that phosphorylates CELF1. Phosphorylation of CELF1 stabilizes the protein and results in its upregulation.[19] Long CUG repeats in DM1 form an imperfect double stranded hairpin structure that sequesters another RNA-binding protein, Muscleblind-like 1 (MBNL1), an homolog of Drosophila muscleblind.[20] MBNL1 co-localizes with CUG RNA nuclear foci in muscle from DM1 patients.[21] Sequestration of MBNL1 by long CUG repeats decreases its activity as a splicing regulator.[22] CELF1 and MBNL1 regulate a number of common splicing events, but in opposite directions.[23] Abnormal splicing observed in DM1 is likely to be the result of the combined effects of loss of MBNL1 activity and upregulation of CELF1 activity.

The expression pattern of CELF proteins across adult and foetal human tissues has been examined by Northern blotting, dot-blot analysis or high-density microarrays.[12-14,24] These studies

revealed that all CELF proteins were expressed at similar levels in all adult brain areas examined and throughout development. CELF3 and CELF5 are preferentially expressed in the brain and some ubiquitous proteins (e.g., CELF2 and 4) are expressed at a high level in the brain. CELF3 appears early in development and is expressed in brain and testis only.[12,13,25] The CELF3 transcript is translated into a bona fide ~50 kDa protein in the brain.[26] In the mouse, CELF4 is brain-specific and is expressed in neurons at a particularly high level in the pyramidal neurons of the CA2 and CA3 regions.[27] Although little information is available to date, neuronal CELF proteins appear to be important in maintaining neuronal function. In *Caenorhabditis elegans*, mutations in *unc-75*, the orthologue of CELF3, CELF4 and CELF5 result in sprouting defects in specific subpopulations of motor neurons accompanied by a locomotor phenotype that could be rescued by expression of human CELF4.[28]

Table 1. CELF proteins and their alternative nomenclature

Protein	BRUNOL nomenclature	Alternative name
CELF1	BRUNOL2	CUG-BP1 NAB50 NAPOR EDEN-BP
CELF2	BRUNOL3	ETR-3 CUG-BP2 NAPOR-2
CELF3	BRUNOL1	TNRC4 ETR-1 CAGH4 ERDA4 MGC57297
CELF4	BRUNOL4	
CELF5	BRUNOL5	
CELF6	BRUNOL6	

Implication of CELF Proteins in Triplet Repeat Expansion Neurodegenerative Disorders

A number of inherited neurological disorders are caused by translated or untranslated repeat expansions in the disease gene. Current evidence points out to an, at least partial, implication of toxicity at the RNA level in most, if not all, of these disorders.[29]

DM1, caused by a CTG repeat expansion in the 3'-UTR of the *DMPK* gene, is primarily a muscle wasting disease, but, in addition, aged patients develop a neurodegenerative phenotype.[30] Neurodegeneration in DM1 is associated with aggregation of the tau protein into neurofibrillar lesions reminiscent of the pathology of Alzheimer's disease.[31,32] A similar tau pathology has been reported in a patient with myotonic dystrophy, type 2 (DM2), caused by an intronic CCTG tetramer repeat expansion in the *ZFN9* gene.[33] Alternative splicing of tau exon 10 (E10) generates tau isoforms with three (3R tau, E10-) or four (4R tau, E10+) microtubule-binding repeats in the C-terminus of the protein; alternative splicing of exons 2 (E2) and 3 (E3) produces tau isoforms with no, one or two inserts in the N-terminus. 3R tau and 4R tau are expressed at approximately equal levels in adult human brain. The pathogenic importance of tau splicing in dementia is demonstrated by a number of FTDP-17 *MAPT* mutations affecting cis-elements controlling E10 splicing and resulting in aberrant 4R/3Rtau isoform ratios.[34] Levels of E2-containing tau transcripts are reduced in cortical neurons in DM1[31,32] and misregulation of E10 splicing has also been reported.[35] Interestingly, tau E2 inclusion is repressed in vitro by CELF2.[36] Also, inclusion of E10 is promoted at high efficiency in vitro by CELF3 and CELF4.[26,37] Thus, an abnormal activity of CELF proteins, including CELF2, 3 and 4, on tau splicing may be a factor contributing to neuronal pathology of DM1. Repression of tau E2/3 inclusion can be obtained by RNAi downregulation of MBNL1 in HeLa cells, suggesting that altered MBNL1 activity may also contribute to changes in tau splicing occurring in DM1.[38] Thus, like muscle and heart transcripts, abnormal tau splicing observed in DM1 might result from the combined effect of loss of MBNL1 activity and upregulation of CELF protein activity. Many transgenic mouse models of DM1 have been generated, and some tau abnormalities in the brain have been reported.[39] It should be noted that, if aberrant splicing is central to tau pathology in DM1, it might be difficult to model in animals as the adult pattern of tau splicing differs between humans and mice.[40]

Fragile X syndrome is an X-linked form of inherited mental retardation, with mild to moderate cognitive and behavioural deficit but no neuronal loss. Fragile X syndrome is caused by expansion over 200 and hypermethylation of a CGG repeat domain in the 5'-UTR of the *FMR1* gene, resulting in its silencing and the loss of its protein product, the FMRP protein, itself an RNA-binding protein.[41,42] Carriers of CGG repeat expansions in the *FMR1* gene, larger than 60, but below the disease threshold of 200 (so called pre-mutations) develop in old age a specific neurodegenerative syndrome referred to as fragile-X-associated tremor/ataxia syndrome (FXTAS). FXTAS is characterized by parkinsonism, brain atrophy and the presence of intranuclear inclusions in neurons and astrocytes.[43,44] The levels of *FMR1* mRNA are elevated by at least five times the normal levels in FXTAS individuals suggesting the involvement of an RNA gain-of-function mechanism.[45,46] In support of an RNA-dependent toxic mechanism, transgenic mice[47] or transgenic Drosophila[48] expressing 90 CGG RNA repeats, within the premutation range, develop a neurodegenerative phenotype. Expanded CGG repeat RNA associated with FXTAS form intranuclear inclusions in transfected cells that recruit several RNA-binding proteins, including MBNL1 at late stages of their formation.[49] MBNL1 also colocalizes with inclusions in FXTAS patients.[50] Overexpression of hnRNP A2/B1 and CELF1 suppresses the degenerative eye phenotype induced by 90 CGG RNA repeats in Drosophila.[51] HnRNP A2/B1 interacts directly with CGG RNA repeats but CELF1 exerts its effect through its interaction with hnRNP A2/B1.[51] The mechanism of FXTAS would therefore bear of similarities with DM1 in that expanded triplet repeat RNA might alter the activity specific RNA-binding proteins, eventually affecting the processing of their targets.[52]

A number of inherited progressive neurodegenerative diseases are caused by CAG repeat expansions in the disease gene translated into a long polyQ tract in the protein product. An increase above approximately 35 in the number of CAG repeats defines the threshold between normal and disease phenotypes. PolyQ-expansion diseases include several spinocerebellar ataxias (e.g., SCA1, 2, 3, 6, 7, 17), Huntington's disease and spinal and bulbar muscular atrophy.[53,54] A screen for modifiers of the neurodegenerative phenotype induced by polyQ-expanded ataxin-3, the SCA3 protein, in Drosophila identified an insertion in the promoter of the *muscleblind* (*mbl*) gene that enhanced photoreceptor neurodegeneration.[55] Accordingly, overexpression of muscleblind as well as human MBNL1 enhanced toxicity. Furthermore, expression of a long untranslated CAG repeat induced neuronal degeneration; conversely, expression of the polyQ sequence through a mixed CAG/CAA sequence had a reduced toxicity.[55] Another study suggested that RNA was unlikely to be involved to a major extent in polyQ expansion diseases.[56] However, the effect of muscleblind on toxicity argues in favour of a role for RNA in the pathogenicity of CAG repeat disorders. Of note, MBNL1 interacts with CAG repeat RNA and co-localizes with CAG repeat RNA foci in cultured cells.[22] Furthermore, a transcriptomic analysis revealed a three-fold downregulation of CELF2 in SCA3 transgenic mice as compared to non-transgenic controls.[57] A trans-dominant effect of CAG repeat RNA might be elicited through abnormal processing of muscleblind targets. Considering that CELF1 and MBNL1 share a number of RNA targets, one might expect, as in FXTAS, that upregulation of CELF1 would reduce the SCA3 phenotype in flies, however, this remains to be tested. It is interesting to note that one member of the CELF family, CELF3, (also know as Trinucleotide Repeat Containing 4 (TNRC4)) contains a 15-18-repeat long polyQ sequence in its divergent domain[12,58]. The CAG repeat of the TNRC4 gene shows little variation in the normal human population and has not been associated with any pathological condition.[59]

Implication of CELF Proteins in Other Neurological Disorders

Generation of transgenic mouse lines expressing an unrelated gene led to the identification of a line, named the frequent-flyer *(Ff)* mouse, developing seizures in heterozygous mutants.[27] Mapping of the insertion in *Ff* mice revealed that the transgene was inserted in intron 1 of the *Celf4* gene.[27] The contribution to *Celf4* disruption to the *Ff* phenotype was confirmed through the generation of *Celf4* knock-out mice which have a phenotype resembling that of the *Ff* mice.[27] Absence of CELF4 results in downregulation of several genes involved in excitability[27] but whether the splicing of specific transcript is affected is yet to be determined.

Spinal muscular atrophy (SMA) is an autosomal recessive disorder of childhood caused by the homozygous deletion of the *survival motor neuron* (*SMN*) gene and characterized by muscular atrophy and weakness resulting from the degeneration of lower motor neurons.[60] The *SMN* gene encodes SMN, a 38 kDa protein with both cytoplasmic and nuclear localizations involved in various aspects of RNA processing.[61] In the nucleus, SMN plays a role in the assembly of snRNP and regulates pre-mRNA splicing.[62] In the cytoplasm, SMN has been shown to be necessary for the transport of specific mRNAs in the axon, such as actin mRNA.[63,64] SMN interacts with a number of RNA-binding proteins; in particular CELF2 interacts with SMN in mouse brain and localizes with SMN in the nucleus of NSC-34 cells.[65] Furthermore, CELF2 is upregulated in muscle cultures from SMA patients as well as in several mouse models of SMA.[65] Anderson et al.[65] suggested that the interaction of CELF2 with SMN together with its high expression in the neuromuscular system could partially explain the detrimental effect of SMN deficiency to motor neurons.

Neurofibromatosis type 1 (NF1) is characterized by the development of tumours of the nervous system: neurofibromas and schwannomas in the peripheral nervous system and gliomas and astrocytomas in the central nervous system.[66] NF1 patients also display cognitive impairment and learning disabilities.[67] NF1 is caused by loss-of-function mutations in the *NF1* gene, encoding neurofibromin, a tumor suppressor with homologies with GTPase activating proteins (GAP) that represses the Ras kinase. Exon 23a in *NF1* encodes a portion of the GAP domain and is differentially spliced between neurons and non-neuronal tissues. Mice with a targeted deletion in exon 23a display a cognitive impairment reminiscent of the phenotype observed in patients with *NF1* mutations.[68] All CELF proteins, with the exception of CELF6, promote exon 23a exclusion in cultured cells.[69] Thus CELF proteins are involved in the regulation of a neurofibromin exon that might be important in regulating higher cognitive functions.

Conclusion

Although a number of neurological disorders involve altered RNA processing, most of them have not been directly linked to clear cis- and trans-acting effects, unlike those seen in FTDP-17 or ALS. A notion that has emerged recently is that proteins causing similar diseases may be part of networks of interacting proteins and genetic modifiers, as shown for SCAs, implying that diseases of the same class can share molecular mechanisms.[70] Interestingly, a number of components of the SCA network are RNA-binding proteins, adding weight to the general implication of abnormal RNA processing in neurological, and particularly neurodegenerative, disorders.[70] Most disease-causing proteins are yet to be assigned to functional networks, however, it could be speculated that CELF proteins/MBNL1 would be consistent components of such networks. Nonetheless, the implication of CELF proteins in many neurological diseases generalizes the pathogenic importance of impaired RNA processing in neurological diseases. In many cases, impaired RNA processing would not be causative, but could contribute to specific aspects of the phenotype of individual conditions.

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